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Review Article

Abundance estimation with DNA metabarcoding – recent advancements for terrestrial arthropods

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Abstract

Biodiversity is declining at alarming rates worldwide and large-scale monitoring is urgently needed to understand changes and their drivers. While classical taxonomic identification of species is time and labour intensive, the combination with DNA-based methods could upscale monitoring activities to achieve larger spatial coverage and increased sampling effort. However, challenges remain for DNA-based methods when the number of individuals per species and/or biomass estimates are required. Several methodological advancements exist to improve the potential of DNA metabarcoding for abundance analysis, which however need further evaluation. Here, we discuss laboratory, as well as some bioinformatic adjustments to DNA metabarcoding workflows regarding their potential to achieve species abundance estimation from arthropod community samples. Our review includes pre-laboratory processing methods such as specimen photography, laboratory methods such as the use of spike-in DNA as an internal standard and bioinformatic advancements like correction factors. We conclude that specimen photography coupled with DNA metabarcoding currently promises the greatest potential to achieve estimates of the number of individuals per species and biomass estimates, but that approaches such as spike-ins and correction factors are promising methods to pursue further.

Key words: abundance, biodiversity monitoring, *COI*, insects, metabarcoding, spike-ins

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Introduction

Biodiversity is declining at alarming rates worldwide (Díaz et al. 2020). The startling observation of a decline in over 75% flying insect biomass in German nature reserves over 25 years (Hallmann et al. 2017) triggered an earthquake in society and politics and raised awareness of arthropod declines, which have since been further documented (Lister and Garcia 2018; Seibold et al. 2019; Simmons et al. 2019; van Klink et al. 2020). Subsequently, numerous initiatives have been launched or reinforced at global to European and regional scales to assess arthropod diversity and also define guidelines for applied, large-scale biodiversity monitoring schemes (Seibold et al. 2019; Ronquist et al. 2020; Potts et al. 2021). Monitoring programmes are frequently limited in spatial coverage

and sampling effort, since they often rely on morpho-taxonomical analysis for species identifications, which is costly and time-consuming (Yu et al. 2012) and additionally limited by a shortage of taxonomic expertise (Fernandes et al. 2019; Watts et al. 2019; Darby et al. 2020; van Klink et al. 2022a). Thus, in order to meet the increased demand for arthropod diversity assessments, traditional morpho-taxonomy approaches need to be combined with other methods (Pawlowski et al. 2018; Compson et al. 2020).

DNA-based approaches offer a promising alternative to arthropod diversity surveys and monitoring (Porter and Hajibabaei (2018); Zinger et al. (2020); Suppl. material 1). In particular, DNA metabarcoding enables high sample throughput (Elbrecht and Steinke 2018; de Kerdrel et al. 2020), due to automation of laboratory and bioinformatic workflows (Krehenwinkel et al. 2017a; Buchner et al. 2021; Buchner et al. 2023) and offers a time- and cost-effective approach for large-scale biodiversity assessments (Piper et al. 2019; Watts et al. 2019). Molecular methods further have the potential to resolve cryptic species (Sow et al. 2019) and intraspecific genetic diversity (Elbrecht et al. 2018) and open up the possibility to include degraded and non-invasively collected material, e.g. faeces (Andriollo et al. 2019), or plant material in biodiversity surveys, which yields high potential for trophic interaction and food web analysis.

However, implementation in policy-mandated monitoring programmes is still hampered (Blancher et al. 2022; Kelly et al. 2023). Reasons for the limited application include general scepticism among taxonomists, missing expertise and infrastructure within state monitoring agencies, a lack of standardised molecular protocols (Dickie et al. 2018; Pawlowski et al. 2018; Zinger et al. 2019; Compson et al. 2020; Creedy et al. 2021), as well as incomplete reference databases (Watts et al. 2019; van der Heyde et al. 2020; Zenker et al. 2020) and the destruction of specimens for DNA extraction (Zizka et al. 2019), although non-destructive approaches are gaining ground (Castalanelli et al. 2010; Carew et al. 2018; Zenker et al. 2020; Batovska et al. 2021; Kirse et al. 2023). The most important shortcoming concerns the limitation to assess the number of individuals per species and biomass, which is essential in standardised monitoring and ecological analysis, but still remains one of the greatest challenges for high-throughput DNA-based approaches (Compson et al. 2020).

Several factors within the metabarcoding workflow affect extraction of abundance data (Pawlowski et al. 2018; Zinger et al. 2019). Firstly, sample properties such as complexity seem to affect abundance information (Piñol et al. 2019). This complexity refers in particular to variation in biomass across and within species (Elbrecht and Leese 2015; Elbrecht et al. 2017; Braukmann et al. 2019), but also marker gene copy numbers (Krehenwinkel et al. 2017b). Secondly, methodological biases skew abundance and biomass estimations. During DNA extraction, a protocol-dependent taxonomic bias can be introduced due to variations in species size and morphology, causing differences in isolated DNA yields (Krehenwinkel et al. 2017a; Pornon et al. 2017; Matos-Maraví et al. 2019; Iwaszkiewicz-Eggebrecht et al. 2022). Several steps in the metabarcoding laboratory workflow, such as PCR amplification and sequencing, can introduce stochastic processes affecting read counts (Leray and Knowlton 2017; Shirazi et al. 2021). Arguably, the strongest bias is caused by taxon-specific differences in primer binding efficiency (Piñol et al. 2015; Krehenwinkel

et al. 2017a, 2017b). The magnitude of primer bias depends on the number of mismatches between primer and target sequence, especially towards the 3‘-end of the primer (Piñol et al. 2019). Apart from primer choice, additional PCR bias can be caused by variable GC content in the target genetic marker (Nichols et al. 2018), amplicon length (Krehenwinkel et al. 2017b) or the occurrence of pseudogenes (Andujar et al. 2021). Thirdly, post-laboratory steps in the bioinformatic processing of sequencing data can skew final read distribution (Frøslev et al. 2017; Alberdi et al. 2018; Darby et al. 2020; Matos-Maraví et al. 2019; Creedy et al. 2021).

A meta-analysis targeting 22 DNA metabarcoding studies revealed a weak relationship between biomass and generated read counts, with a large degree of uncertainty (Lamb et al. 2019). The studies included in Lamb et al. (2019) used different protocols and a wide range of target organisms and sample types, which somewhat hampers overall comparability, but does emphasise that raw read counts are not suitable to infer abundance estimates.

A variety of different approaches have emerged recently that can help improve abundance and biomass estimates from metabarcoding data, including species-specific correction factors applied to read counts, spike-ins, primer optimisation or multi-locus metabarcoding (e.g. Richardson et al. (2015); Krehenwinkel et al. (2017b); Richardson et al. (2019); Darby et al. (2020); Luo et al. (2023); Suppl. material 1). However, these advances have so far not been compared systematically for complex arthropod samples. Furthermore, since taxonomic biases exist in DNA metabarcoding data (Clarke et al. 2014), some form of species-specific correction of DNA metabarcoding would be required to obtain robust estimates of individuals per species. This may not be required to reliably estimate total biomass, but still requires the conversion of read counts to biomass, which is not trivial.

Here, we review potential methods that can improve abundance and biomass estimation in arthropod whole organism community (WOC) samples. Considering the variety of approaches and applications, we aim to formulate general recommendations for DNA metabarcoding workflows in arthropod monitoring. In addition, we explore approaches from metabarcoding studies targeting e.g. aquatic samples that have so far not been applied to terrestrial arthropods and their trophic interactions.

Methods

Collection of relevant literature and assessment of methodological approaches

We performed an online literature search in Google Scholar and EBSCO Discovery Service on 17 January 2022 using the keywords [(quant*) AND (insect) AND (metabarcod*) AND (DNA)] and included only peer reviewed publications in English. Although the search term specifically targeted insects, we use the more general term “terrestrial arthropods” throughout the text. Additionally, some publications were added to the list based on the authors’ expertise.

We included studies that applied DNA metabarcoding to terrestrial arthropods as target organisms and/or in relation to their trophic interactions within ecosystems (e.g. pollination and food web studies), as these topics are strongly

connected and play an important role in monitoring schemes (e.g. ecosystem services of pollination or natural pest control). With these criteria, WOC and tissue samples were included covering also pollen, gut contents and faeces as well eDNA metabarcoding approaches, such as extraction from soil and sample fixative. We excluded studies that applied individual-based DNA barcoding and next generation sequencing (NGS) barcoding, PCR-free approaches as well as long-read sequencing methodologies, as we wanted to focus on metabarcoding specifically. PCR-free approaches are, however, briefly discussed in an outlook section.

Based on 113 publications matching our search criteria (Suppl. material 2), we extracted information on article type, study type, sample type, species group, methods and parameters (Table 1, Suppl. material 3). We examined these methods regarding their applicability to study types (species richness assessments, pollen analysis, food web studies) and to sample types (WOC samples, pollen, eDNA and gut contents/faeces). The overall suitability was assessed based on whether certain abundance metrics (number of individuals per species, relative abundance) as well as biomass estimation were achievable, whilst also considering the extent of additional equipment, cost and labour (Suppl. material 4). These considerations are based on the available literature.

Abbreviations:

ASV	amplicon sequence variant
ddPCR	digital droplet PCR
eDNA	environmental DNA
FOO / POO	frequency of occurrence / percent of occurrence
NGS / HTS	next generation sequencing / high-throughput sequencing
qPCR	quantitative PCR
RRA	relative read abundance
UMI	unique molecular identifier
WOC samples	whole organism community samples

Results

Description and assessment of methods

Reviewing the literature, we identified three main methods to estimate species abundance with metabarcoding (Table 1): (i) semi-quantitative metrics (Fig. 1), (ii) approaches that can potentially reduce read abundance biases (Fig. 2), and (iii) the combination of DNA (meta-)barcoding with other methodological approaches, which we present in more detail in the following sections (also see Suppl. material 1). This review focuses on studies including developments associated with the laboratory workflow. For a critical assessment of missing standards in bioinformatics we refer to Creedy et al. (2021). Since many metabarcoding studies refer to relative abundances, whilst monitoring aims to determine counts of individuals per species, we make a clear distinction of these terms throughout this manuscript by referring to “number of individuals per species” (absolute number of individuals belonging to the same

Table 1. Overview of methodological approaches discussed in this publication.

Category	Approach	Sample types	Quantitative?
Semi-quantitative metrics	FOO/POO	all	semi-quantitative
	RRA	all	semi-quantitative
	rarefaction	all	semi-quantitative
	transformation	all	semi-quantitative
Reducing read abundance biases	correction factors via algorithm	WOC samples	yes; virtual specimen counts
	correction factors via mock communities	all	yes
	spike-ins	all	yes; relative abundance
	primer optimisation	all	no
	multi-locus metabarcoding	all	yes
Combination of methods	general	all; depending on approach	yes; depending on approach
	photography and body measurements of single specimens	WOC samples	yes; number of individuals per species, biomass

species) and "relative abundance" (proportion of a species within a sample), but use the more general term "abundance" as a summary term. "Biomass" (weight of individuals belonging to the same species) is mentioned separately, where appropriate.

Semi-quantitative metrics

DNA metabarcoding is comprehensively used to assess presence/absence from complex sample mixtures. Whilst this can be informative for some ecological assessments, including biodiversity measures (e.g. alpha diversity), interaction analyses (e.g. multi-trophic networks, food web structures, plant-pollinator interactions) require some form of (semi-)quantitative data. There are different approaches to conduct semi-quantitative analysis of DNA metabarcoding data (Fig. 1A). In diet analyses, frequency or percentage of occurrence (FOO/POO; Fig. 1B) are often applied (Deagle et al. (2019), but see Cuff et al. (2022)). In bipartite networks, link strength (Fig. 1C) is a meaningful quantitative metric for plant-pollinator or prey-predator networks (Cuff et al. 2022; Thomsen and Sigsgaard 2019). Alternatively, relative read abundance (RRA) summarised over biological replicates is often used (Fig. 1D), especially for pollen samples (Kratschmer et al. 2019; Wilson et al. 2021). Various studies have applied read counts, RRA as well as derived metrics, such as log- or rank-transformed or rarefied read abundance to assess community composition for different sample types (pollen, faeces, gut and WOC samples; Hope et al. (2014); Hawkins et al. (2015); Richardson et al. (2015); Krehenwinkel et al. (2018); Macías-Hernández et al. (2018); Marquina et al. (2019)). The use of any of the above-mentioned metrics is straightforward, but in most cases they are unreliable for quantitative inferences due to the various factors affecting read counts (Pawlowski et al. 2018; Zinger et al. 2019) and thus they are uninformative to estimate the number of individuals per species or biomass.

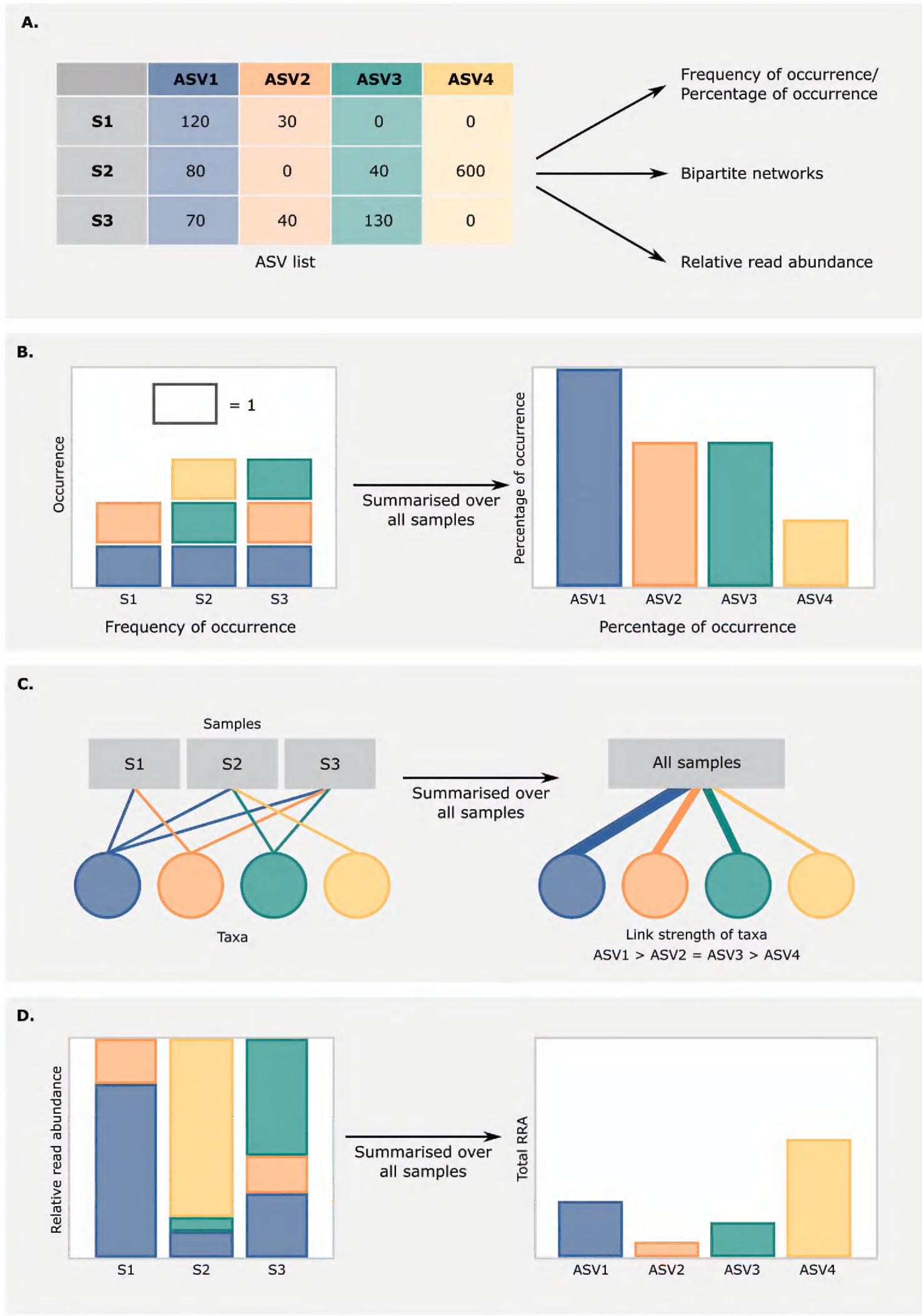

Figure 1. Semi-quantitative metrics. **A** ASV table as the outcome of a DNA metabarcoding experiment, rows are samples, columns are ASVs, numbers are raw read counts. From the ASV table, semi-quantitative metrics can be derived, e.g. frequency and percentage of occurrence, bipartite networks and relative read abundance. **B** Frequency and percentage of occurrence derived from ASV table, frequency of occurrence simplifies the ASV table into presence/absence data, indicated by presence or absence of a rectangle (left), when summarising this over all samples, percentage of occurrence can be an informative metric for abundance in a system (right). **C** Bipartite networks derived from the ASV table, samples and ASVs are nodes, edges indicate presence/absence of the ASVs per sample (left), when summarising this over all samples, link strength can be an informative metric for abundance in a system (right). **D** Relative read abundance derived from ASV table, relative read abundance for individual samples is determined by dividing raw read counts of individual ASVs by total read count per sample (left), when summarizing this over all samples, mean relative read abundance can be an informative metric for abundance in a system (right); abbreviations: S – Sample, ASV – Amplicon sequence variant, RRA – relative read abundance; ASVs are colour coded and refer to ASVs from (**A**), artwork: Alice Scherges.

Approaches that reduce read bias

Correction factors

Mock community experiments have shown a positive correlation of read counts per species with genomic template DNA concentration in pollen and WOC samples (Baksay et al. 2020; Gueuning et al. 2019), while other studies revealed a PCR bias introduced through taxon- and marker-specific primer efficiency (Bell et al. 2019; Braukmann et al. 2019; Darby et al. 2020; Krehenwinkel et al. 2017b). Since these biases are strongly affected by primer binding efficiencies (Piñol et al. 2019), they are assumed to be predictable (Krehenwinkel et al. 2017b). Thus, correcting read counts using species-specific correction factors can improve metabarcoding-derived abundance estimates (Krehenwinkel et al. (2017b); Darby et al. (2020); Fig. 2A, B). Such correction factors can be obtained using mock communities (Krehenwinkel et al. (2017b), Fig. 2A) or iterative "guess-and-test" algorithms (see further down, Darby et al. (2013); Darby et al. (2020); Table 1, Fig. 2B, Suppl. material 1). In order to derive correction factors using mock communities, artificial community samples of defined composition are processed alongside unknown samples. However, the derived correction factors can only be applied to species that are present both in environmental and artificial community samples, which is a strong limitation for hyperdiverse WOC arthropod samples such as Malaise trap catches that contain many unknown taxa.

It may be possible to extend correction factors to closely-related taxa based on phylogenetic relatedness, whereby similar skews of read counts are assumed. In microbial analyses (Goberna and Verdu 2016), such an approach has been used to infer functional traits. In beetles, a recent paper has shown a significant correlation between species biomass and high-throughput sequencing (HTS) read abundance for *16S*, but not for *COI*, which had more primer-template mismatches. In addition, strong phylogenetic signals in primer-template mismatches were identified and models incorporating the effects of mismatch type or number improved species biomass estimation from HTS read abundance for *COI* (Liu et al. 2023). To obtain correction factors, mock communities have so far only been used in combination with WOC arthropod samples (Krehenwinkel et al. 2017b), but this approach could also be transferred to pollen samples, as processing mock communities alongside such samples is common (Bell et al. 2019; Baksay et al. 2020; Swenson and Gemeinholzer 2021). Species-specific correction factors obtained from mock community samples are helpful to reduce read abundance biases; however, some sources of bias still exist, e.g. related to the evenness of a community sample (Piñol et al. 2019), copy number variations of the target gene (Krehenwinkel et al. 2017b) or differences in DNA quality between specimens used for mock community samples versus field-collected samples (Krehenwinkel et al. 2018). Furthermore, it should be noted that correction-factors obtained via mock communities are expected to fluctuate in response to changes in PCR cycle numbers across metabarcoding workflows (Yang et al. 2020; Martoni et al. 2022).

Correction factors can also be calculated using an iterative algorithm which mitigates data skews due to copy number variations of the target gene (Darby et al. (2020, 2013); Fig. 2B, Suppl. material 1). This requires a reference dataset

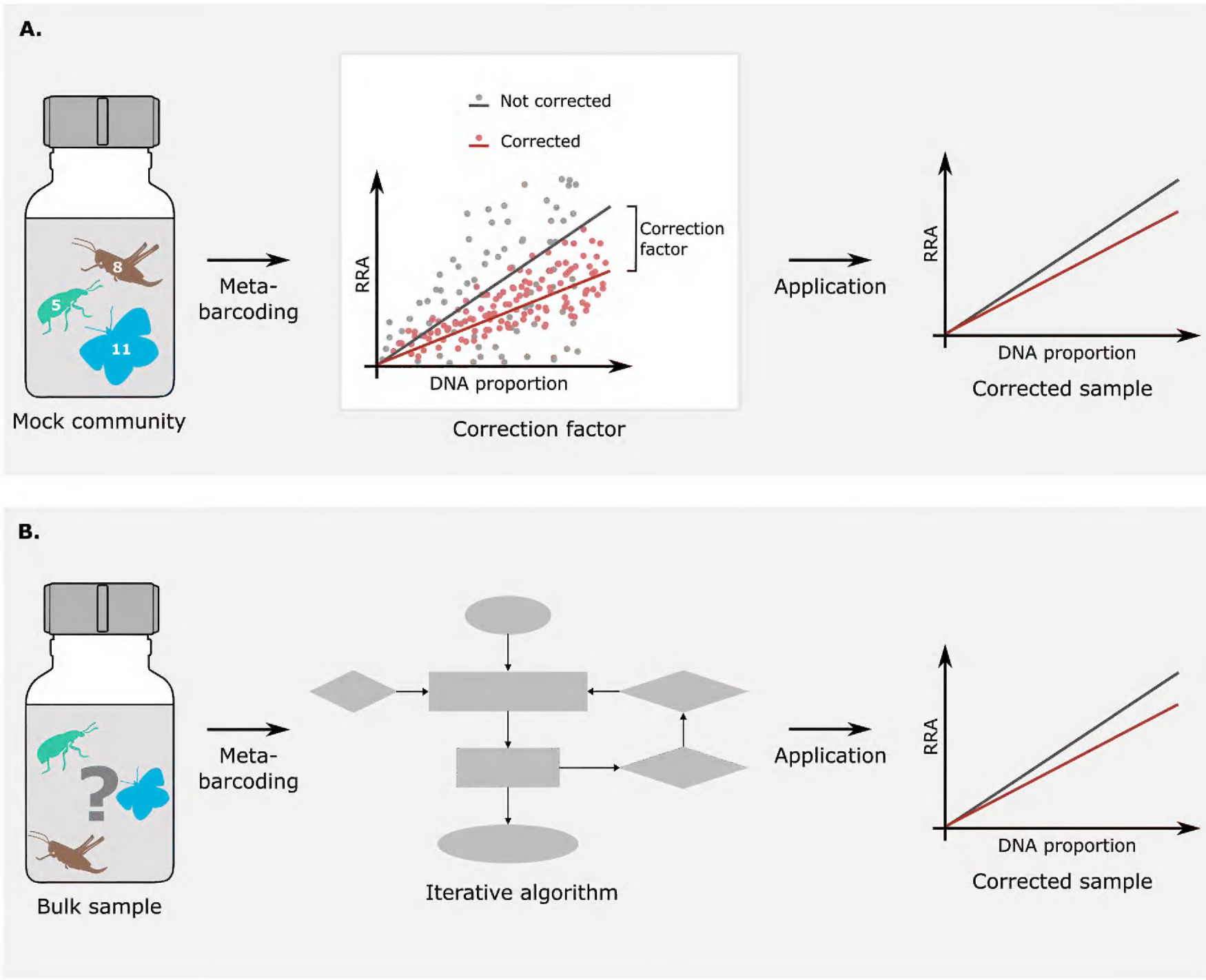

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Figure 2. Reducing read abundance biases. **A** Processing mock communities (bottle) with defined composition allows determining taxon-specific correction factors, which can be applied to correct relative read abundance of samples with unknown composition, indicated by a red line. Correction factors can only be determined for taxa included in the mock community. **B** Correction factors can be determined using iterative algorithms and a guess-and-test approach based on a morphological reference data set (not shown). The correction factors can be applied to correct relative read abundance of samples with unknown composition, indicated by a red line. Correction factors can only be determined for samples that show a good agreement in terms of taxa detected between the reference and the DNA metabarcoding data set. **C** Adding spike-ins, e.g. a defined amount of genomic DNA, to all samples and co-amplifying and co-sequencing the reference material allows correcting raw read counts by simply dividing read counts assigned to taxa (blue and brown bars) by read counts assigned to the spike-in (red bars); abbreviations: RRA – relative read abundance, S – sample, artwork: Alice Scherges.

with specimen counts obtained from morphological identifications. The algorithm itself then starts with randomly generated correction factors for each species to compute predicted specimen counts from metabarcoding data. The predicted counts are compared to the reference data set and correction factors are iteratively adjusted until predicted and actual counts converge (Darby et al. 2013).

The algorithm can only be applied to samples with high concordance between morphological and DNA-based taxonomies, but it is a promising approach, as the predicted numbers of individuals per species were highly correlated with actual count data (Darby et al. 2013; Darby et al. 2020). It requires high-quality material and specimens to be identified morphologically (Darby et al. 2013; Darby et al. 2020) and thus can only be used for WOC and tissue samples (see Suppl. material 1). Time and cost of the overall analysis increases, as a reference set of morphologically identified species is required, but this could be worth it in the case of repeated monitoring of sites with known species composition, or for the monitoring of known arthropod pests.

Spike-ins

Spike-ins (Fig. 2C) may also be referred to as internal standards (ISDs; Harrison et al. (2021)). Here, a defined amount of reference material DNA is added to each sample, which allows read count correction and thereby improves abundance and biomass estimation (Luo et al. 2023). The reference DNA can be added as tissue (Darby et al. 2020), genomic DNA, pre-amplified DNA (Ji et al. 2020), plasmids (Luo et al. 2023) or synthetic DNA (Palmer et al. 2018), should include primer binding sites and needs to be added to the reference database (Tkacz et al. 2018; Luo et al. 2023). Spike-ins are added to the samples in a standardised manner, e.g. a defined amount of reference DNA (ng) per defined volume of lysis buffer (µl; Ji et al. (2020); Luo et al. (2023)). It is recommended to add the spike-in after tissue lysis but prior to DNA extraction (Ji et al. 2020; Luo et al. 2023), so that it is co-extracted, co-amplified and co-sequenced along with the sample DNA and therefore underlies the same methodological biases. Since all samples receive the same amount of spike-in, they should theoretically return the same spike-in read counts. However, sample complexity affects read numbers (Piñol et al. 2019) and thus different samples will return different read numbers for the spike-in (Luo et al. 2023). Read correction can be achieved by dividing the number of reads assigned to amplicon sequence variants (ASVs) by the number of reads assigned to the spike-in, resulting in significant improvement in within-species abundance across samples (Ji et al. 2020; Luo et al. 2023).

The use of spike-ins is not restricted by sample type, but comes with a low increase in effort and costs, because the spiking of samples is an additional, albeit minimal, step in the laboratory workflow, which has to then be integrated in the bioinformatic workflow. It should be noted that spike-in correction does not correct for biases across species within samples (Luo et al. 2023). The underlying reasons have, to our knowledge, not been systematically addressed, but may very well relate back to sample complexity and primer binding efficiencies (Piñol et al. 2019). It has been proposed that species-specific correction factors obtained from mock communities (see previous section), unique molecular identifiers (UMIs, see outlook section) as well as the application of less biased primers can be used

to correct for within-sample across species biases (Ji et al. 2020; Luo et al. 2023). Spike-in correction is a straightforward and powerful approach with high potential to improve abundance and biomass estimations via DNA metabarcoding.

Primer optimisation

A variety of studies have shown that primer design is an essential part determining the success of DNA metabarcoding studies, both in terms of taxon recovery and read abundance biases (Esnaola et al. 2018; Jusino et al. 2018; Lafage et al. 2019; Pedro et al. 2020). Primers used in DNA metabarcoding need to be universal and the fragment length needs to be suitable for the sequencing platform of choice, whilst allowing for species-level identification (Meusnier et al. 2008). Over and under amplification of different lineages of arthropods (Krehenwinkel et al. 2017b; Darby et al. 2020) as well as certain plant species dominating pollen samples (Bell et al. 2019; Baksay et al. 2020) have been reported and should be minimised as much as possible. Thus, primer design, including primer validation and evaluation, is a painstaking task, which needs to be continuously pursued using complex mock communities to ensure the best choice of primer for arthropod metabarcoding studies (Elbrecht et al. 2019).

Multi-locus metabarcoding

Different genetic markers suffer from different taxonomic biases and thus some studies employ several different loci for the same organismal group, which is referred to as multi-marker (Adamowicz et al. 2019) or multi-locus metabarcoding (Batovska et al. 2021). Multi-locus metabarcoding has been applied to WOC and tissue samples (Marquina et al. 2019; Giebner et al. 2020), pollen (Richardson et al. 2015; Bell et al. 2019; Richardson et al. 2019), faecal samples and gut contents (Swift et al. 2018; Krehenwinkel et al. 2019; Gil et al. 2020) as well as soil and even eDNA samples (Ritter et al. 2019; Thomsen and Sigsgaard 2019).

Locus-specific biases can be mitigated by using rank order abundance or median-based proportional abundance summarised over all loci, as has been demonstrated in pollen DNA metabarcoding (Richardson et al. 2015; Richardson et al. 2019). The locus-specific PCRs are often performed separately (Richardson et al. 2015; Swift et al. 2018; Richardson et al. 2019; Baksay et al. 2020; Darby et al. 2020), which increases time and cost for sample processing. Multi-locus metabarcoding can be performed in multiplexed reactions (de Kerdrel et al. 2020; Batovska et al. 2021) to improve time and cost efficiency. However, this may introduce additional read abundance skews, possibly due to PCR competition between loci (Batovska et al. 2021). During analysis, data from different markers need to be analysed separately (Thomsen and Sigsgaard 2019), which increases time for analysis. It should be emphasised that different markers usually yield discordant taxon lists (Alberdi et al. 2018; da Silva et al. 2019), e.g. because of incomplete reference databases for markers other than *COI* (Andujar et al. 2018), but may also be attributed to differences in PCR efficiency. Such discordant taxa lists allow a broader taxon coverage, but it also means that data from different markers are complementary (Kirse et al. 2021), complicating data analysis. In the case of discordant taxa lists, abundance estimates (e.g. rank-based) can only be determined for taxa identified by more than one marker (Richardson et al. 2015; Richardson et al. 2019).

For pollen samples, as no single universal plant barcode exists (CBOL Plant Working Group 2009; Bell et al. 2016), multi-locus metabarcoding was commonly adopted especially in early studies (Kraaijeveld et al. 2015; Richardson et al. 2015; Pornon et al. 2016). Consequently, pollen DNA metabarcoding workflows include both ribosomal (e.g. *ITS2*) and chloroplast markers (e.g. *trnL*, *rbcL*, *matK*) (Richardson et al. 2015; Milla et al. 2021; Swenson and Gemeinholzer 2021; Arstingstall et al. 2023). The latter generally perform well concerning PCR amplification and taxonomic resolution, despite concerns regarding little chloroplast DNA in pollen grains (Hawkins et al. 2015; Kraaijeveld et al. 2015; Bell et al. 2016). There even exists some evidence that chloroplast markers are more suitable for assessing relative abundances than ribosomal markers (Richardson et al. 2019; Baksay et al. 2020), possibly due to lower GC contents (Baksay et al. 2020), and these differences need to be carefully considered. However, more recently, pollen DNA metabarcoding studies may revert to single-locus metabarcoding, focusing on *ITS2* (Leidenfrost et al. 2020), due to incomplete reference databases for and sub-optimal taxonomic resolution of chloroplast markers (Richardson et al. 2019; Kolter and Gemeinholzer 2021).

Combining DNA metabarcoding with other methods

Some studies combine DNA metabarcoding with other methodologies. Thereby, DNA metabarcoding may be used to obtain a comprehensive species list of the detected taxa, whilst abundance estimates (e.g. number of individuals per species, DNA copy number) and/or biomass estimates are obtained with another methodology. One common example is the complementary morphological analysis of gut content remains, pollen grains or arthropod specimens (Keller et al. 2015; Darby et al. 2020; Gil et al. 2020). Other examples are weighing WOC samples (Hausmann et al. 2020), using flow cytometry of pollen (Baksay et al. 2020) or other forms of PCR (Schneider et al. 2016; Tedersoo et al. 2019). The choice of additional methodology determines the sample types that can be used, for example, combining metabarcoding with quantitative PCR (qPCR; Schneider et al. (2016)) or digital droplet PCR (ddPCR; Tedersoo et al. (2019)) can be performed on all sample types. For other methodologies, for example weighing, WOC samples are required (Hausmann et al. 2020). All these data sources are complementary and can provide different kinds of information, e.g. total biomass of WOC samples obtained from weighing, supplemented by species-level presence/absence data provided by metabarcoding (Hausmann et al. 2020).

One noteworthy approach of method combination is the photographic documentation of specimens from WOC samples before analysing them with DNA metabarcoding. This combined approach enables individual counts, body size measurements and thereby biomass estimation (Gueuning et al. 2019). As specimens are handled individually (Wührl et al. 2022), the use of body parts for DNA extraction, instead of full specimens, is furthermore facilitated (Gueuning et al. 2019; Darby et al. 2020), keeping voucher specimens mostly intact. Specimen photography further allows documentation of specimens for future reference as well as incorporating a pre-sorting strategy (Elbrecht et al. 2020). Whilst handling of individual specimens is exceptionally time- and labour-intensive, automated solutions can improve time-efficiency (Ärje et al. 2020; Wührl et al. 2022). In combination with machine learning approaches, the

automated screening of high-resolution pictures of arthropod WOC samples for abundance estimation is emerging and would facilitate large-scale assessments, e.g. for monitoring schemes (Høye et al. 2021). While these approaches are still in development, the vision of completely automated protocols, incorporating image recognition before molecular sample processing, exists (Høye et al. 2021; Besson et al. 2022; van Klink et al. 2022b; Wührl et al. 2022). However, exactly when highly accurate image recognition to species level for all arthropod species in a WOC sample might be possible and thereby circumvent the need for metabarcoding altogether, is difficult to assess.

Discussion

General conclusions and recommendations for arthropod monitoring and related questions

The available literature has revealed that the majority of (terrestrial) arthropod DNA metabarcoding studies do not sufficiently address the matter of estimating the number of individuals per species and/or biomass (Suppl. material 2). In terms of pollen analysis, research exists that discusses abundance estimation via DNA metabarcoding, but with inconsistent results (Keller et al. 2015; Kraaijeveld et al. 2015; Richardson et al. 2015; Bell et al. 2019; Richardson et al. 2019; Baksay et al. 2020). In contrast to this, DNA metabarcoding has received considerably more attention in the aquatic sector in recent years and advancements exist both for WOC samples of macrozoobenthos and eDNA (Elbrecht and Leese 2015; Elbrecht et al. 2017; Beentjes et al. 2019; Hoshino et al. 2021). Existing policies, like the EU Water Framework Directive (WFD; Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; Directive 2008/56/EC), legally require routine monitoring of aquatic environments. As a consequence, standards for sampling, processing and reporting already exist (Haase et al. (2004), but see Birk et al. (2012)), as well as DNA-based indicators (Aylagas et al. 2014). Especially the DNAqua-Net COST Action (Leese et al. 2016; Leese et al. 2018) has published many advancements regarding the suitability and integration of (e)DNA metabarcoding in biomonitoring (Buchner et al. 2019; Pawlowski et al. 2018), as well as resources to facilitate standardisation and quality control for DNA-based monitoring (Bruce et al. (2021); Bruce and Keskin (2021); Vasselon et al. (2021); Leese et al. (2023), DNAqua-Hub, https://dnaquahub.eu/; accessed 24 May 2022). This work has a high potential to be transferred into terrestrial arthropod monitoring and demonstrates that DNA metabarcoding can indeed be standardised for monitoring purposes (Leese et al. 2023), which is far more challenging for morphological species identification. However, this transfer could be hampered by the lack of data on diversity and distributions of the hyperdiverse arthropods, although such studies are now emerging (Buchner et al. 2023; Srivathsan et al. 2023).

Additionally, the collected literature focused on approaches that apply to the sample processing stage of metabarcoding workflows. The effect of bioinformatics and data analysis strategies on abundance and biomass estimations is strongly underrepresented (Suppl. material 2). A variety of non-harmonised bioinformatic tools and pipelines exists (Creedy et al. 2021), but a more detailed discussion on the bioinformatics and data analysis side of this topic is outside the scope of this review. However, future research needs to address this.

As expected, there is a variety of adjustments attempting to improve abundance and biomass estimation via DNA metabarcoding (Suppl. material 2). It remains, however, difficult to find a "one-size-fits-all" approach to assessing individual counts and biomass from DNA metabarcoding, partly because different approaches are applicable only to certain sample types or because recent advancements still do not translate to individual counts and/or biomass estimates.

Overall suitability of DNA metabarcoding approaches to estimate the number of individuals per species and biomass in terrestrial arthropod monitoring

Currently, the most promising approach is to combine DNA metabarcoding with specimen photography, which would ideally be automated (Ärje et al. 2020; Wührl et al. 2022). In addition, promising avenues such as correction factors and spike-ins should be further developed (Darby et al. 2013; Krehenwinkel et al. 2017b; Darby et al. 2020; Ji et al. 2020; Luo et al. 2023). Specimen photography coupled with automatic image recognition facilitates body size measurements to achieve biomass estimates as well as the number of individuals per species. Combining the approaches of Gueuning et al. (2019), Darby et al. (2020) and de Kerdrel et al. (2020) seems especially promising, as recombining specimens to "pseudo-community" samples allows cost-efficient mixed-species DNA (meta-)barcoding. We would like to point out that this strategy is not the same as NGS barcoding (Wang et al. 2018; Srivathsan et al. 2021), since individual specimens or parts of them are combined to mixed-species samples (Gueuning et al. 2019; de Kerdrel et al. 2020). Thus, samples are processed following a metabarcoding workflow, but obtained barcodes can be traced back to specimens (de Kerdrel et al. 2020). We argue that despite the increase in processing time and associated costs, (automated) specimen photography is a simple and effective way to achieve considerable improvement in taxon recovery, as well as estimates regarding the number of individuals per species and biomass (Fig. 3). This approach is limited to WOC samples, although a similar approach may potentially be applied to pollen samples, for example by flow cytometry (Baksay et al. 2020; Dunker et al. 2020). Theoretically, these approaches could be combined to achieve count and biomass data, although the above-mentioned studies did not comment on this potential.

Regardless of application or sample type, general recommendations for every metabarcoding workflow are to use appropriate positive controls, i.e. mock communities (Ji et al. 2020), as well as negative controls, biological and technical replicates (Alberdi et al. 2018; Elbrecht and Steinke 2018; Liu et al. 2019; Zinger et al. 2019; Yang et al. 2020) and consider multi-locus metabarcoding. Each of these steps can improve taxon detection and the correlation between relative read abundances and input DNA mass (Richardson et al. 2019; Ritter et al. 2019; Thomsen and Sigsgaard 2019; Ji et al. 2020). Associated increases in costs and labour are justified by the improvement in the generated data, although budget limitations may deem technical replicates unfeasible. With optimised metabarcoding and bioinformatic workflows, more robust relative abundance and biomass estimates are thus potentially achievable in the foreseeable future. However, the number of individuals per species cannot be determined, as other sources of bias still exist. We therefore recommend considering additional approaches discussed further down.

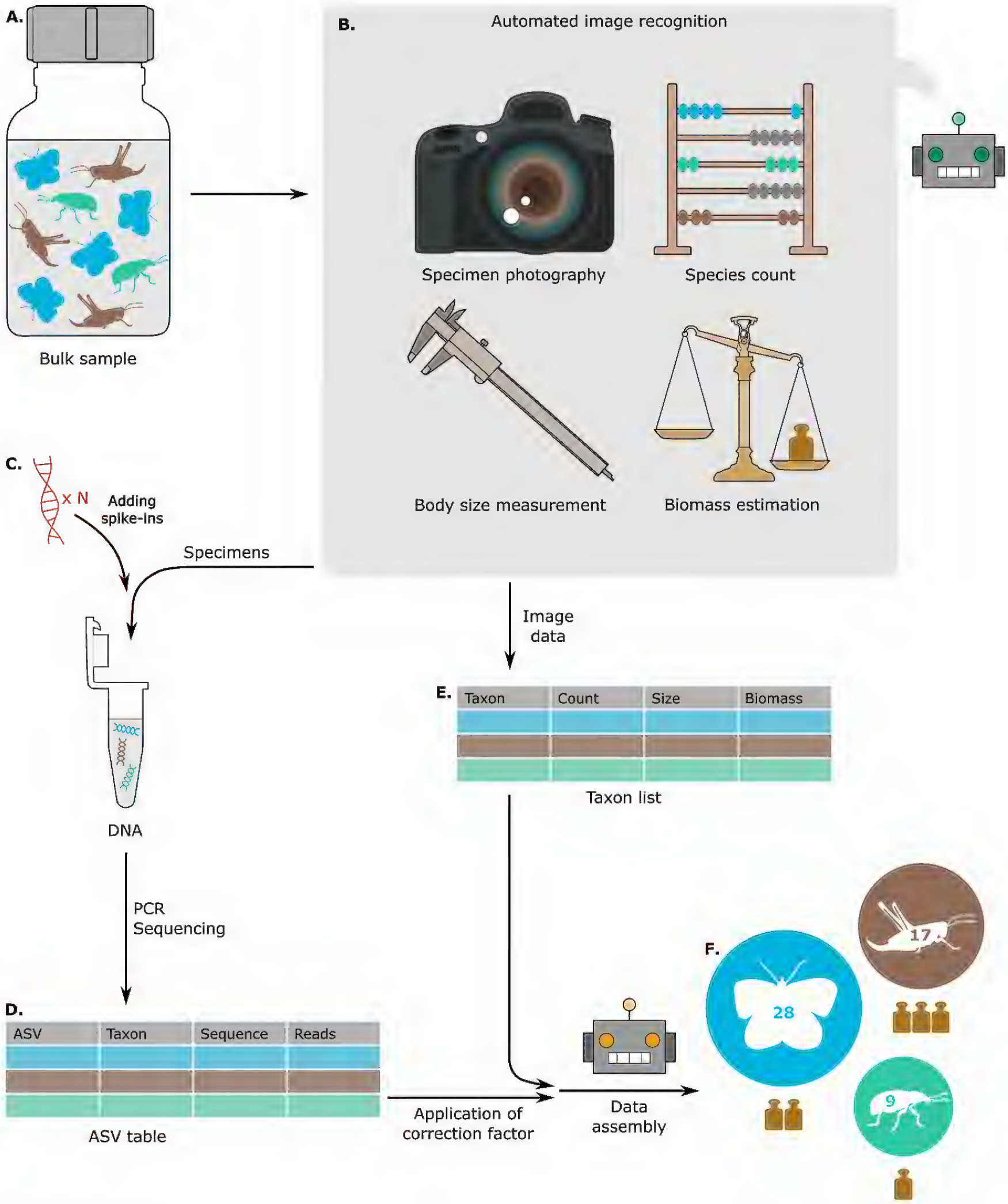

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Figure 3. Recommended workflow for biodiversity assessments with bulk samples and DNA metabarcoding that obtains count and biomass data with species level taxonomic identifications. **A** Specimens from a bulk sample (bottle) are first processed individually. **B** Processing includes specimen photography (camera), specimen counts (abacus), body size measurements (caliper) and biomass estimation (scales). Ideally, this is done automatically (green robot icon) and involves automatic image recognition to achieve preliminary taxa identifications on broad taxonomic scales. **C** Specimens are then re-combined to a community sample, a spike-in is added and DNA is extracted (microcentrifuge tube). **D** DNA metabarcoding delivers species level identifications and raw read counts (ASV table), which are corrected via the spike-in. **E** Image data is combined to a taxon list containing count, size and biomass data (taxon list). **F** Image data and DNA metabarcoding data are combined using machine learning approaches (data assembly, orange robot icon) to obtain a data set that contains information on species level identities, along with count data and biomass estimates (taxa bubbles), abbreviations: ASV – amplicon sequence variant, artwork: Alice Scherges.

For eDNA, obtaining count data is extremely difficult. Since eDNA dynamics (Barnes and Turner 2016; Compson et al. 2020) are affected by various uncontrollable factors prior to sampling, analysis of abundance information is further impeded. Thus, presence/absence and derived frequency or percentage of occurrence (FOO/POO) data from replicates currently seem to be the best option, although promising approaches exist that will move towards more informative data obtainable from eDNA. For example, combining species detections with information about the cellular and molecular state of eDNA (e.g. intra- versus extra-cellular eDNA, genetic region, fragment size) is expected to improve the abundance estimation, as demonstrated in water samples (Jo et al. 2021). Other options for eDNA-based monitoring are: an overall experimental design and sampling strategy that allows indirect counts, developing and applying novel metrics (e.g. the ”eDNA index”; Kelly et al. (2019)) or coupling presence/absence data with site-occupancy models (van Strien et al. 2010; van Strien et al. 2013). We argue that eDNA approaches are worth considering for arthropod monitoring, as they are non-invasive (Andriollo et al. 2019; Thomsen and Sigsgaard 2019; Pumkaeo et al. 2021; Roger et al. 2022), which is especially important for protected and endangered species.

Additionally, (e)DNA-based analyses open up new avenues that move away from the traditional estimation of numbers of individuals per species or biomass. One such avenue to pursue further is more sensitive detection rates of parasitism and invasive species (Sow et al. 2019; Young et al. 2021). Thus, (e)DNA metabarcoding deserves to be incorporated in such schemes at least as a complementary approach to morpho-taxonomy.

Outlook: Further molecular approaches for the estimation of species abundances

In the following, we explore selected approaches from the wider literature that were not within the scope of the present review. However, there is high potential for the implementation in monitoring programmes in the future. Novel data analysis pipelines are constantly being developed and some focus on integrating uncertainties associated with the dynamics of DNA in the environment (Barnes and Turner 2016; Compson et al. 2020). One such example, a tracer model, has successfully been applied to estimate the abundance of target fish species (Fukaya et al. 2020). Another example, an “eDNA index”, which is a double-transformation of read-counts, holds potential to assess abundance trends across time and space (Kelly et al. 2019). Additionally, species occupancy models can detect false negatives (Compson et al. 2020) and Bayesian hierarchical models can integrate primer choice and other parameters of the metabarcoding workflow (Doi et al. 2019; Compson et al. 2020), which would allow correcting read count-derived abundance estimates. Lastly, the application of half-life corrections and prey DNA decay rates allow the inference of relative frequencies and biomass of prey items based on metabarcoding and shotgun sequencing data (Uiterwaal and DeLong 2020; Paula et al. 2023).

When grouping sequencing reads as ASVs instead of molecular operational taxonomic units, DNA metabarcoding can potentially deliver conservative abundance estimates in the sense of “minimum census estimates”, similar to those obtained from non-invasive sampling of hair and faeces (Frantz et

al. 2004; Miotto et al. 2007). In this case, the evolutionary rate of the chosen marker would have to be considered (Wang et al. 2016), as it may affect the recovery of ASVs per species and consequently the obtained minimum census estimates. Furthermore, ASV-based analyses facilitate the analysis of intraspecific diversity (Elbrecht et al. 2018; Arribas et al. 2021; Shum and Palumbi 2021; Weitemier et al. 2021), which is greatly underappreciated in arthropod monitoring schemes. Another promising approach is to further refine the qSeq protocol (Hoshino and Inagaki 2017; Hoshino et al. 2021) and similar workflows employing unique molecular identifiers (UMIs; Luo et al. (2023)). Here, a single-primer extension is included in the workflow before performing PCR. During this step, each DNA fragment is labelled with a random tag and the number of random tags per ASV can be used to accurately infer starting copy numbers of each recovered sequence in the original sample. This allows simultaneous species identification and inference of relative abundances from eDNA and WOC samples (Hoshino and Inagaki 2017; Hoshino et al. 2021; Luo et al. 2023). Unique molecular identifiers have also been applied in detecting rare allele variants and mutations and have been reported as being especially useful for read error corrections (Jabara et al. 2011; Kinde et al. 2011; Kivioja et al. 2012; Fields et al. 2020).

There is an urgent need to shift away from a purely morpho-taxonomic approach and related indicators for long-term arthropod monitoring, towards an integrative framework, in which morphological and molecular biological methodologies are applied in parallel. This requires the development and implementation of novel proxies and indicators to indirectly assess species abundance based on genetic data. One possible approach is to apply Hill numbers to DNA-based and morpho-taxonomic assessments alike, as this improves comparability and they can even be applied to (phylo-)genetic data (Alberdi and Gilbert 2019). Additionally, the amount of genomic DNA per taxon can reliably be assessed by combining metabarcoding with qPCR or ddPCR, although these usually focus on specific target species (Schneider et al. 2016; Tedersoo et al. 2019), but also via the use of UMIs (see above).

PCR-free methods represent a further alternative (Garrido-Sanz et al. 2020; Ji et al. 2020; Cordier et al. 2021). The advantage of these approaches is that no amplification step is conducted and therefore, the complete mitochondrial or nuclear DNA is sequenced and analysed. As PCR amplification is omitted, mito- and metagenomic approaches are associated with more reliable abundance estimations. Ideally, whole mitochondrial or nuclear reference genomes of target taxa exist in order to assign generated reads to the species of origin (Schmidt et al. 2022; Theissinger et al. 2023). However, the absence of reference genomes for non-model organisms poses a barrier to the application of PCR-free approaches (Formenti et al. 2022; Lewin et al. 2022) and thus *de novo* assemblies (Meng et al. 2019) may be a suitable alternative, especially for mitogenomics. The general suitability of mito- or metagenomics for large-scale arthropod monitoring, however, remains limited due to higher costs, computing power and data storage requirements. Further, bias introduced through extraction and variable gene copy number still exist in those approaches. Nonetheless, genomic approaches are valuable and informative for biodiversity conservation purposes, as they enable more detailed analyses of intra-specific diversity and population structure than DNA (meta-)barcoding allows (Theissinger et al. 2023).

Concluding remarks

Even though there are many details to consider when applying DNA metabarcoding to arthropod monitoring, pollen and food web analyses, we were able to make some general recommendations. Generally, DNA metabarcoding should always be optimised for maximum taxon recovery and minimal amplification biases. The processing of adequate positive and negative controls is essential. Incorporating appropriate biological and technical replicates reduces the impact of certain methodological biases.

DNA metabarcoding as a rapid tool to obtain species occurrences is a very promising method for large-scale monitoring activities, especially when abundance estimates are not required. When combining DNA metabarcoding with specimen photography and body size measurements, the number of individuals per species and biomass can also be assessed.

Going forward, creating new DNA-based metrics to report (relative) abundances based on genetic units rather than processing individual specimens offers new innovations addressing the most central questions in arthropod monitoring, as these rarely require absolute measures of abundance. Detecting and assessing trends in monitoring relates more to within- and between-sample comparisons taken across spatial and temporal scales, which can be achieved with metabarcoding. Additionally, DNA metabarcoding facilitates the assessment of ecosystem services in a time- and cost-efficient manner, via processing pollen and food web analyses.

There are still many challenges to face until metabarcoding data can deliver robust abundance and biomass estimations. Currently, sorting and individual handling of specimens from WOC samples is unavoidable to obtain such data. However, it is important to apply both classical morpho-taxonomy and molecular biological approaches in parallel, which will allow the management and analysis of the large amounts of data generated by monitoring programmes in a timely and cost-effective manner. Thus, despite its limitations, DNA metabarcoding can and should be incorporated as an additional tool in routine arthropod monitoring to increase sample sizes and cover a broader range of taxonomic groups.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

WS and PD devised the study. WS performed the literature review and drafted the first version of the manuscript. WS, PD, VZ and SJB were substantially involved in subsequent drafts. AS created the figures. All authors agreed to the final version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Background information

Authors: Wiebke Sickel, Vera Zizka, Alice Scherges, Sarah J. Bourlat, Petra Dieker

Data type: docx

Explanation note: Background information on DNA metabarcoding and methodological adjustments to improve abundance and biomass estimates.

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Link: https://doi.org/10.3897/mbmg.7.112290.suppl1

Supplementary material 2

Literature collection

Authors: Wiebke Sickel, Vera Zizka, Alice Scherges, Sarah J. Bourlat, Petra Dieker

Data type: xlsx

Explanation note: Relevant publications were identified in two steps: 1) via an online literature search in Google Scholar and EBSCO Discovery Service; keywords: ((quant*) AND (insect) AND (metabarcod*) AND (DNA)), including only peer-reviewed publications in English, results were screened for suitability based on title and abstract; 2) addition of publications based on the authors' expertise; included studies that applied metabarcoding with arthropods and/or in relation to their trophic interactions within ecosystems (e.g. pollination, food web studies); excluded topics were: individual-based DNA barcoding and NGS barcoding, long-read sequencing methodology, mito-/metagenomics and genome skimming.

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Link: https://doi.org/10.3897/mbmg.7.112290.suppl2

Supplementary material 3

Categories assessed in the literature review

Authors: Wiebke Sickel, Vera Zizka, Alice Scherges, Sarah J. Bourlat, Petra Dieker

Data type: xlsx

Explanation note: For each category, possible parameters are given, together with examples and more detailed explanation where appropriate and necessary.

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Supplementary material 4

Evaluation of methodological approaches

Authors: Wiebke Sickel, Vera Zizka, Alice Scherges, Sarah J. Bourlat, Petra Dieker

Data type: xlsx

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